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Effect of Reduced N^6-Benzyladenine, Explant Type, Explant Orientation, Culture Temperature and Culture Vessel Type on Regeneration of Adventitious Shoot and In Vitro Plantlets of *Spilanthes acmella*

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Nodal explants of *Spilanthes acmella* produced normal multiple shoots when cultured vertically on Murashige and Skoog medium (1962) supplemented with 0.5 mg L^{-1} BA. The number of shoots formed from each explant was doubled after first 5-week subculture. The nodal explants placed vertically in Erlenmeyer flask (250, 500, or 1000 mL) produced more multiple shoots than those cultured in 350 mL jam bottles and 500 mL tex-Z flask. Temperature above 28°C caused abnormalities of in vitro plantlets. All in vitro plantlets survived after acclimatized and transferred to the outside environment. The survived plantlets did not show any morphological abnormalities in the field condition.

Keywords: apical shoot, in vitro plantlets, multiple shoot, nodal segments, *Spilanthes acmella*

Malaysia has unlimited resources and abundance of plants that provide important and useful secondary metabolites in its rainforests. Plants have been used as sources of drugs, food additive, fragrance and pesticides (Morris et al., 1985; Staba and Zito, 1985; Broussalis et al., 1999; Ng, 1999). More than 2000 species of plants have been documented to possess insecticidal properties (Klocke, 1989; Broussalis et al., 1999). The secondary metabolites that these plants produce do play an important role in the defence barrier against pest. However, the potential utilization of these plants as a source of insecticide has rarely been explored though a number of them have been traditionally used for insect pest control (Luthria et al., 1993). The genus *Spilanthes* consists of 42 known species and several insecticidal compounds have been reported in *Spilanthes mauritiana, Spilanthes alba, Spilanthes ocymyfolia, Spilanthes oleracea* and *Spilanthes acmella* (Burkill, 1966; Krishnaswamy et al., 1975; Borrges-del-Castillo et al., 1984; Jondiko, 1986; Cook, 1996; Ramsewak et al., 1999).

S. acmella is an annual herb belonging to the Compositae family. It can be found in the moist soil of Malaysian wasteland and disturbed forest. All parts of the plants are acrid but the flowers are by far the most pungent. It has been well documented for its uses as spices, antiseptic, anti-bacterial, anti-fungal, anti-malarial and as remedy for toothache, flu, cough, rabies diseases and tuberculosis (Burkill, 1966; Oliver-Bever, 1986; Di Stas et al., 1994; Akah and Ekekwe, 1995; Singh, 1995; Storey and Salem, 1997; Ramsewak et al., 1999). The plant is said to be a popular remedy for stammering in children in western India. It is also used in the treatment of the dysentery and rheumatism (Baruah and Leclercq, 1993). There are reports that *S. acmella* contains alkaloids that have the potential to act as an insecticide (Krishnaswamy et al., 1975; Borges-del-Castillo et al., 1984) and were found to be able to control *Aedes aegypti* in Kenya (Jondiko, 1986).

Today, much research has been done on the chemical analysis and structure determination of pungent alkamides from *S. acmella*. To the best of our knowledge, none of the published paper proposed an in vitro mass propagation of this plant for the production of active compounds. These plants were successfully micropropagated using axillary buds as explants by Ang et al. (2000). The authors reported that a few factors such as the explant type, explant orientation, type of culture vessels and the growth substance combination were observed to be affecting the adventitious shoot formation and the regeneration of the in vitro plantlets of *S. acmella*. The objective of this study is therefore to analyze in depth these effects and to establish the optimum conditions for regeneration of normal in vitro plantlets of *S. acmella* and to be used as plant materials for the production of bio-insecticides.

MATERIALS AND METHODS

Establishment of Aseptic Plant Materials

S. acmella was collected from the herbal garden of

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Plant Tissue and Cell Culture Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The axillary buds of the plant were used as explant for producing the in vitro plantlets. The explants were first washed with detergent and rinsed with distilled water. They were then dipped into 70% alcohol for one minute before surface sterilized with 0.08% (w/v) $HgCl_2$ for 5 minutes. After rinsing with sterile distilled water, the explants were sterilized with Clorox® 15% (v/v) for 15 minutes and followed by rinsing three times with sterile distilled water. The explants were dried on sterile filter paper before inoculating onto basic MS culture medium (Murashige and Skoog, 1962) without any hormones. The pH of the medium was adjusted to 5.7-5.8, followed by addition of 7.5 g L^{-1} Difco Bacto agar before autoclaving. All the cultures were incubated at 25 ± 2°C under continuous light provided by white fluorescent light with a light intensity of 32.5 μE m^{-2} s^{-1}. After two weeks, the aseptic explants were transferred onto the MS agar medium supplemented with 2.0 mg L^{-1} N^6-Benzyladenine (BA) (Ang et al., 2000). The established proliferating cultures of *S. acmella* were used as the source of explants for the subsequent experiments.

Effect of Reduced BA on Adventitious Shoot Regeneration

To study the effect of reduced BA concentration (0.5-2.5 mg L^{-1}) on formation of multiple shoots in *S. acmella*, the axillary buds of *S. acmella* plantlets were cultured on MS supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1} BA. Thirty explants were used for each medium treatment and the experiment was repeated three times. The number of shoots formed from each explant and shoot length were recorded after five weeks of culture and again after the first subculture. The data were analysed using one-way Analysis of Variance (ANOVA) and the best medium was selected after comparison of mean values by Duncan Multiple Range test (DMRT) at p=0.05.

Effect of Explant Type and Explant Orientation on Adventitious Shoot Regeneration

Apical shoots (3.0-5.0 mm in length) and single nodes (10.0 mm in length) were dissected out from the in vitro plantlets of *S. acmella*. They were cultured in 250 ml Erlenmeyer flask containing MS supplemented with 0.5 mg L^{-1} BA, the optimum medium for multiple shoot formation as investigated above. Both types of explant were placed in two orientations on the culture medium, either vertically or horizontally. Ten experimental units were carried out for each explant type and each explant orientation. Each flask contained three explants and all the cultures were incubated in the same condition as mentioned above. After 5 weeks of culture, the number of shoot formed and the length of shoot were recorded. The data were analysed using ANOVA with 2x2 factorial design and the comparison of means was performed with Tukeys Studentized Range (HSD) Test at p=0.05.

Effect of Vessel Type and Explant Orientation on Adventitious Shoot Regeneration

Single nodes (10.0 mm in length) were cut from in vitro plantlets and cultured in 350 mL jam bottle, 500 mL tex-Z flask, and Erlenmeyer flask (250, 500 or 1000 mL) containing MS medium with 0.5 mg L^{-1} BA. Each type of culture vessel had the explants oriented in two positions on the culture medium, either vertically or horizontally. Ten experimental units were used for each type of culture vessel and each explant orientation. Each experimental unit contained three explants. The cultures were placed in a culture room under continuous light with cool white fluorescent tubes at an intensity of 32.5 μE m^{-2} s^{-1} at 25 ± 2°C. After 5 weeks culture, the number of shoot formed from each explant and the length of shoot were recorded. The data were analysed using ANOVA with 2x2 factorial design and the means were compared using Tukey's Studentized Range (HSD) Test at p=0.05.

Temperature Effect on In Vitro Plantlets of *S. acmella*

Single nodes (10.0 mm in length) were cut from in vitro plantlets and cultured in 250 mL Erlenmeyer flask containing MS medium supplemented with 0.5 mg L^{-1} BA. All the cultures were subjected to three different temperature conditions (24°C, 28°C, and 31°C). Ten experimental units were used for each temperature condition. After 5 weeks culture, the number of shoot formed, length of shoot and the percentage of abnormal plantlets were recorded. The data were analysed using one-way ANOVA and the means were compared using Tukeys Studentized Range (HSD) Test at p=0.05.

Acclimatization of *S. acmella* Plantlets

The in vitro rooted plantlets were washed with distilled water to remove adhering culture medium and transferred to Jiffy-7. All plantlets (n=50) were then placed in a plastic container covered with plastic film to maintain high humidity and were incubated in a

growth chamber at 25 ± 2°C under a continuous light at 8.5 μE m^{-2} s^{-1} light density for two weeks. The plastic film was then removed and the plantlets were maintained in the incubator for an additional week before being transferred to a shaded area in the green house. After one week, the acclimatized plantlets in Jiffy-7 were transplanted to the soil. The survival rate of plantlets was recorded after 2 weeks of transferring to evaluate the success rate of the acclimatization process, and growth of plantlets was observed for another 2 months.

RESULTS AND DISCUSSION

MS medium supplemented with 0.5 mg L^{-1} of BA gave highest to regeneration rate of adventitious shoots in *S. acmella,* and an average of 3.4 shoots were formed from each axillary bud in this medium within five weeks. However, the number of shoot formed on MS medium supplemented with BA in the range of 0.5-2.5 mg L^{-1} was found to be not significantly different. As the concentration of BA increased, smaller shoots were formed (Fig. 1). Cellárová and Kimáková (1999) also found that low concentration of BA of less than 1.0 mg L^{-1} was most effective for induction of multiple shoot formation of *Hypericum perforatum* L. Su et al. (2000) reported that MS medium added with low concentration of BA (0.3 mg L^{-1}) produced normal shoot and root in *Typhonium flagelliforme.* Likewise, all the in vitro shoots of *S. acmella* were normal with complete root system when low concentration of BA (0.5 mg L^{-1}) was added in the culture medium. MS medium supplemented with 2.0 mg L^{-1} or more of BA resulted in vitrification of plantlets.

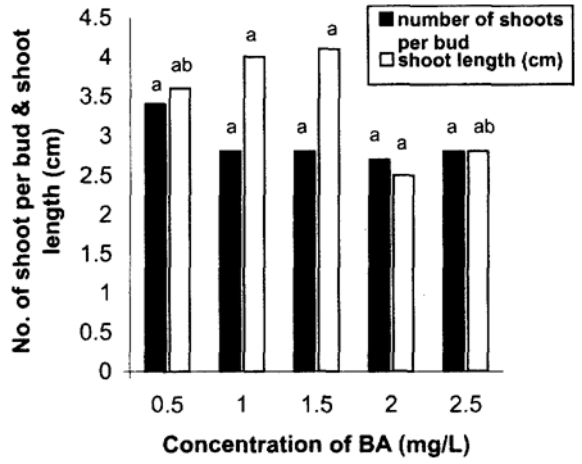

Figure 1. Number of shoots formed per bud and shoot length (cm) of *S. acmella* when cultured on MS medium supplemented with low concentration of BA (0.5-2.5 mg L^{-1}) after 5 weeks of cultivation. Same alphabets on top of each bar indicate that mean values are not significantly different at p=0.05 using HSD.

Number of adventitious shoot formed increased about two fold after the first sub-culture of the separated individual shoots. The shoot length was reduced as the concentration of BA increased in the culture medium (Table 1). Healthy and normal plantlets with multiple shoots complete root system were produced in MS medium supplemented with 0.5 mg L^{-1} BA after the first subculture (Fig. 2) that could be extended for the next round subculture.

The number of shoot produced from single node and apical shoot explant was not significantly different either they were placed vertically or horizontally (Table 2). The apical shoot explants were found to elongate with axillary shoot growing rapidly from each node. This indicated weak apical dominance and easy release of axillary bud as reported by Sudha and Seeni (1996).

Table 1. Number of shoot formed per bud and shoot length (cm) of *S. acmella* when cultured on MS medium supplemented with low concentration of BA (0.5-2.5 mg L^{-1}) after first subculture.

MS + BA (mg L^{-1})	Number of shoot / bud	Shoot length per shoot (cm)
0.5	6.5 ± 1.0 a	5.3 ± 1.0 a
1.0	5.5 ± 1.0 a	4.4 ± 1.1 b
1.5	6.5 ± 2.1 a	4.2 ± 1.0 b
2.0	6.6 ± 1.7 a	3.0 ± 0.6 c
2.5	6.6 ± 1.2 a	3.3 ± 1.0 c

Means within the same column followed by the same alphabet are not significantly different at p=0.05 using DMRT. Standard error is given.

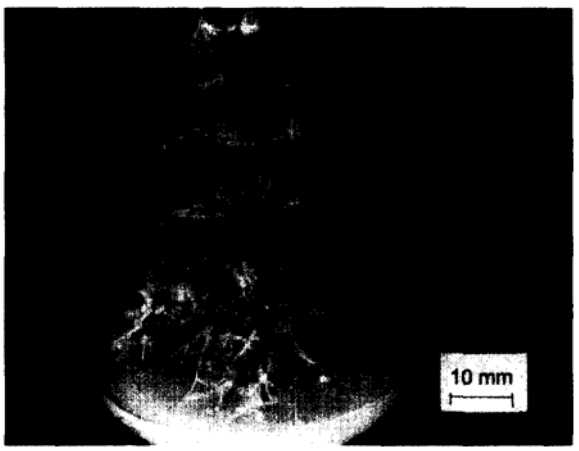

Figure 2. Normal multiple shoot plantlets of *S. acmella* with complete root system after first subculture on MS medium supplemented with BA 0.5 mg L^{-1} in 5 weeks.

Table 2. Effect of explant type and explant orientation on number of shoot formed from each explant and shoot length of *S. acmella* in 5 weeks.

Explant Type	Orientation	Mean number of shoot per explant	Mean length of shoot (cm)
Node	Vertical	1.9 ± 0.04 a	8.9 ± 0.4 a
	Horizontal	1.9 ± 0.05 a	8.9 ± 0.2 a
Apical shoot	Vertical	1.8 ± 0.09 a	6.6 ± 0.3 b
	Horizontal	1.7 ± 0.2 a	5.1 ± 0.5 c

Means within column followed by the same letters are not significantly different at p=0.05 by using HSD. Standard error is given.

Whereas, the length of shoot induced from nodes was significantly increased. The nodal explants responded well and each shoot grew vigorously to reach an average length of 8.0-9.0 cm in 5 weeks. It was reported that proliferation of side shoots from axillary buds, termed 'nodal culture' was a preferred strategy to maintain genetic stability (George, 1993; Cassells and Curry, 2001).

Different culture vessels were found to influence the number of shoot formed from each explant and the height of shoot. However, the orientation of explant, cultured vertically or horizontally, did not affect the multiple shoot formation when they were cultured in 500 mL Tex-Z flask or Erlenmeyer flasks. However, the number of shoot formed from each explant that was cultured vertically or horizontally in the jam bottles was significantly different (Fig. 3). Explants cultured vertically in the 1000 ml Erlenmeyer flask produced the longest shoot length (10.5 ± 0.5 cm) while the explants cultured horizontally in 300 ml jam bottle and 500 ml flask produced the shortest shoot length (Fig. 4). Mackay and Kitto (1998) also reported that different culture vessels had significant effect on shoot length and proliferation rate of micropropagated plants of French tarragon. The effects of vessel types on the shoot multiplication rate might be due to different concentration levels of carbon dioxide, ethylene and other volatile gases in the air space within the container (George and Sherrington, 1984). Studies had showed that explant orientation during incubation could affect shoot induction and proliferation rate of plant such as soybean (*Glycine max* L. Merr.) (Kim et al., 1990) and apple (*Mallus domestica* Borkh.) (Zimmerman and Fordham, 1989).

Higher temperature (31°C) induced more multiple shoot formation but the shoot height was not significantly affected by high temperature. Cultures maintained at 25°C produced normal plantlets, but when

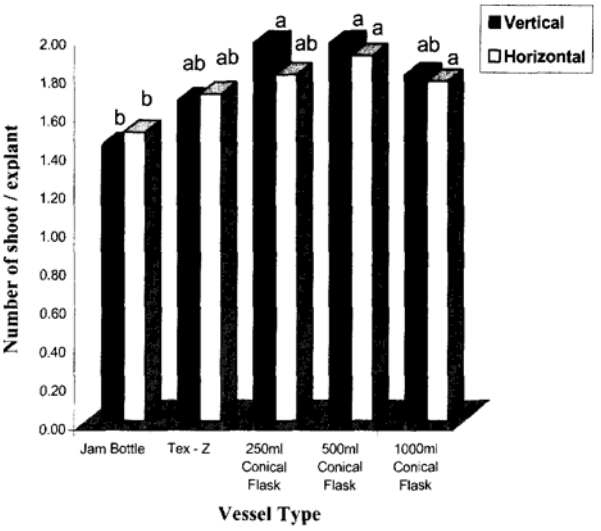

Figure 3. Effects of vessel types and explant orientation on multiple shoot formation of *S. acmella* within 5 weeks. Same alphabets on top of each bar indicate that the mean values are not significantly different at p=0.05 using HSD.

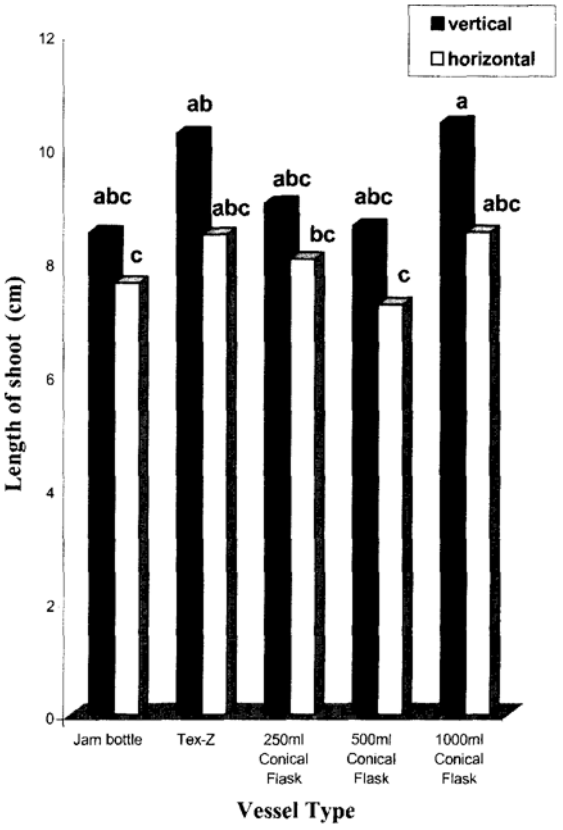

Figure 4. Effects of vessel type and explant orientation on shoot height of *S. acmella* within 5 weeks. Same alphabets on top of each bar indicate that the mean values are not significantly different at p=0.05 using HSD.

Table 3. Effect of different temperature on number of shoot, length of shoot and percentage of abnormal plant after 5 weeks of culture.

Temperature (°C)	Mean number of shoot per explant	Mean length of shoot (cm)	Percentage of abnormal plant (%)
25°C	1.9 ± 0.05 b	6.1 ± 0.3 b	0 c
28°C	1.9 ± 0.1 b	7.3 ± 0.3 ab	6.7 ± 3.7 bc
31°C	2.3 ± 0.2 a	7.0 ± 0.3 ab	19.4 ± 4.4 a

Means within column followed by the same letters are not significantly different at p=0.05 using HSD. Standard error is given.

the cultures were incubated at 28°C and 31°C, and 6.7% and 19.4% of the plantlets became abnormal, respectively (Table 3). The abnormal plantlets were stunted with short internode and twisted shoot. Each single node was accompanied with three leaves rather than two leaves as in the normal plantlets (Fig. 5). However, all the plantlets (normal and abnormal) survived even at these higher temperature, contrary to the report by Sutter and Langhans (1978).

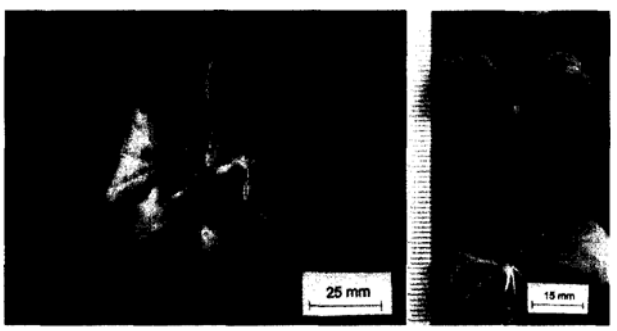

Figure 5. The abnormal plantlets of *S. acmella* with 3 leaves at each node (left) compared with normal plantlet with two leaves at each node (right).

Figure 6. Normal and healthy acclimatized plantlets of *S. acmella*.

The in vitro plantlets in Jiffy® were normal and healthy after the acclimatization process (Fig. 6) and all (100%) survived when transferred to the outside environment. The acclimatization process undertaken was primarily intended for restoring stress resistance and autotrophic competence. The survived plantlets did not show any morphological abnormalities in the field condition.

This study showed that *S. acmella* could be micropropagated on MS medium supplemented with 0.5 mg L^{-1} BA and maintained in a culture room at 25°C. The in vitro plantlets could be used as the plant material for the future study and production of bio-insecticidal compounds.

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